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Production of an extracellular lipase by *Candida utilis* NRRL-Y-900 using agro-industrial by-products

[Tarım sanayi yan ürünlerinden *Candida utilis* NRRL-Y-900 ile ekstraselüler lipaz üretimi]*

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ABSTRACT

Aim:To screen various yeast cultures and agro-industrial by-products and optimization of fermentation conditions for the microbial production of extracellular lipase under solid state fermentation technique.

Material and Methods: Various yeast cultures including *Candida lipolytica* NRRL-Y-1095, *Candida utilis* NRRL-Y-900, *Candida tropicalis* NRRL-Y-1552, *Saccharomyces cerevisiae* IIB-1 were screened by culturing on agro-industrial by-products under batch culture using solid state fermentation in 250 mL Erlenmeyer flasks. The medium was supplemented with various nitrogen sources (both organic and inorganic) and metal ions.

Results: *Candida utilis* NRRL-Y-900 showed the highest enzyme production on soybean meal. Various particle sizes of substrate and moistening agents were also optimized for the maximum lipase synthesis. The optimum temperature and pH for the accumulation of enzyme by *Candida utilis* NRRL-Y-900 was 30ºC and 6.5, respectively. The fermentation time of 60h was suitable for the maximum enzyme production by using 7.5% inoculum of 24h old yeast culture. The optimal medium composition consisted of 2% (w/v) meat extract, 0.4% (w/v) ammonium sulphate and 5mM Fe+2. The maximum extracellular lipase production was 3.96±0.09 U.

Conclusion: The results obtained during the study are significant for, to our knowledge, it is the first report regarding the utilization of soybean meal by *C. utilis* NRRL-Y-900 to accumulate lipase under optimized conditions and solid state fermentation.

Key Words: *Candida utilis*; lipase; solid state fermentation; soybean meal.
Conflict of Interest: The authors do not have any conflict of interest.

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ÖZET

Amaç: Ekstraselüler lipazın katı faz fermantasyon ile mikrobiyal olarak üretilebilmesi için çeşitli maya kültürleri ve farklı tarım sanayi yan ürünleri değerlendirilerek fermantasyon koşullarının optimizasyonu

Gereç ve Yöntemler: *Candida lipolytica* NRRL-Y-1095, *Candida utilis* NRRL-Y-900, *Candida tropicalis* NRRL-Y-1552, *Saccharomyces cerevisiae* IIB-1 dahil bir çok maya kültürü ile tarım sanayi yan ürünleri katı faz fermantasyonu kullanılarak 250 mL erlenmayer içinde seri kültür ile taranmıştır. Vasatlar organik ve inorganik olmak üzere farklı azot kaynakları ve metal iyonları ile beslenmiştir.

Bulgular: En iyi enzim üretimini Soya küspesi ile *Candida utilis* NRRL-Y-900 gösterdi. Koşullar sübstrat olarak değişik parçacık boyutları ve nemlendirici faktörler kullanılarak en fazla lipaz sentezi için optimize edilmiştir. *Candida utilis* NRRL-Y-900 ile enzim birikimi için optimum sıcaklık 30ºC, pH 6.5 dır. Yirmidört saatlik maya kültüründen %7.5 inoculum kullanıldığında 60 saatlik fermantasyon maksimum enzim üretimi sağlamıştır. Optimum vasat kompozisyonu 2% (w/v) et özü, 0.4% (w/v) amonyum sülfat ve 5mM Fe+2 içermektedir. Maksimum ekstracelüler lipaz üretimi 3.96±0.09 U olmuştur.

Sonuçlar: Bildiğimiz kadarı ile bu çalışmanın soya küspesinden *C. utilis* NRRL-Y-900 ile optimize edilmiş katı faz fermantasyonu koşulları altında lipaz akümülasyonu gösteren ilk çalışma olması sebebi ile önemlidir.

Anahtar Kelimeler: *Candida utilis, lipaz, katı faz fermantasyonu, soya küspesi*
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Introduction

Lipases (*E.C. 3.1.1.3*) belong to the hydrolase group of enzymes that act on carboxylic ester bonds. Lipases hydrolyse triglycerides to fatty acids and glycerol and, under certain conditions, catalyse the reverse reaction forming glycerides from glycerol and fatty acids [1]. Lipases can be acquired from many sources such as plants, animals and microorganisms [2]. Extracellular lipase production has been reported by microorganisms including bacteria, fungi and yeasts [3]. However enzyme production is well-known among yeasts. Yeasts are unicellular microorganisms, having certain advantages over bacteria and fungi such as ease of growth and handling and utilization of less refined and cheaper substrate such as agro industrial by-products due to their greater resistance to high substrate concentration and more tolerance to metal ions [4]. Among yeasts, *Candida* sp. has a great potency for the lipase production and utilization of triglycerides [5]. Some *Candida* sp. that produces lipase are *C. curvata*, *C. tropicalis*, *C. valida*, *C. lipolytica*, *C. rugosa, C. utilis and C. pellioculosa* [6]. Although, fermentation conditions have been widely studied but less attention has been paid to the ability of *Candida utilis* to synthesize lipase using agricultural by products [7].

Two common methods used for lipase production are submerged fermentation (SmF) and solid state fermentation [8]. In solid state fermentation (SSF), microbial growth occurs on or near the surface of the moist solid substrate [9]. SSF has several advantages over SmF as it is well adapted and cheaper method [10]. It requires less space, little energy consumption, simplified fermentation media, simple machinery and gives higher yield and high recovery of enzyme. It also has less chances of contamination due to absence of free water [11]. Despite several advantages of SSF, there have been a very few studies on SSF lipase production using yeast [12].

Pakistan, being an agriculture country produces large quantities of agricultural residues each year which find trivial applications such as landfills or burnt off. A number of agro-industrial residues including wheat, rice and barley brans, oil cakes of soy, olive, gingelly and babassu, various oil seed meals and bagasse (sugarcane) have been reported to be capable for the synthesis of lipase [13]. The use of these by-products reduces the cost of the production of lipase.

Culture condition's optimization for maximum lipase accumulation is important, provided these do not affect enzyme properties and enzyme extra to intracellular ratio [14]. Significant factors that affect SSF process are: substrate nature and its properties such as particle size and water holding capacity as well as type of microorganism, period of cultivation and size of the inoculums and the physical parameter including temperature, gaseous atmosphere.

The aim of the present study was to investigate four yeast cultures and to optimize fermentation conditions for the enhanced production of lipase in SSF using different agro-industrial by-products as substrate. To our knowledge, it is the first report regarding the utilization of soybean meal for extracellular lipase production by *Candida utilis* NRRL-Y-900 under solid state fermentation.

Materials and Methods

Microorganisms: Four different yeast strains viz., *Candida utilis* NRRL-Y-900, *Candida lipolytica* NRRL-Y-1095, *Candida tropicalis* NRRL-Y-1552 and *Saccharomyces cerevisiae* IIB-1, obtained from the stock culture of Institute of Industrial Biotechnology (IIB), GC University Lahore, Pakistan were used in the present study. These were maintained on yeast extract peptone dextrose agar (YPD) medium contain (g/L) yeast extract, 10; dextrose, 20; peptone, 20 and agar, 20. The pH was adjusted to 4.5.

Substrates: Various agro-industrial by-products viz., canola meal, almond meal, coconut oil cake, soybean meal, barley bran and wheat bran, were purchased from the local market. Oil cake, oil seed meals and brans were ground to obtain coarse powder before use. Different particle sizes such as 1.5, 2, 2.5, 3 and 3.5 mm were obtained using sieves of various pore sizes.

Inoculum: The yeast cell suspension for inoculation was prepared by adding ten milliliter of slightly warm sterile distilled water to a yeast slant culture having adequate growth under aseptic conditions. To obtain homogenous yeast cell suspension, a sterilized needle was used. A haemocytometer was used to obtain a uniform cell count i.e. 3.5×10^6 cells/mL, in all experiments.

Fermentation technique: The solid substrates (15g) were taken in a series of 250 ml Erlenmeyer flasks, impregnated with 15 ml of distilled water (1:1, substrate, water, ratio) and sterilized at 121°C for 20 min. The same substrate to diluent ratio i.e. 1:1 was used when the substrate was ranged from 5-50g. After cooling, the flasks were inoculated with 1 ml of yeast cell suspension. The contents of the flasks were mixed. All flasks were placed in an incubator at 30°C for 72 h. All the batch culture experiments were run in a set of three parallel replicates.

Determination of water holding capacity: Dried sample of substrate (3-4 g) was mixed with an excess of distilled water and deionized water. After that the substrate was allowed to hydrate for 2 h. The excess of water was removed by permitting the wet sample to drain on a fine meshed wire screen. A portion of the wet sample on the screen was removed, weighed and dried to a constant weight in a vaccum drying oven at 110°C. Water holding capacity (WHC) (in grams of water absorbed by 1 g of dry sample) was calculated as follows [7]:

$$WHC = q_w - q_d / q_d \quad \text{eq 1}$$

Where, q_w = wet weight and q_d = dry weight

Water holding capacity, defined as the ability of the solid substrate to retain water even though external pressures

are applied to it, is known to play an important role in the SSF.

Analytical techniques

Enzyme extraction: At the termination of the fermentation process, 100 mL of distilled water was added in each flask and incubated at 30°C for 1 h. The contents of the flask were filtered through Whatman filter paper no 1 followed by centrifugation for 15 min at 4500 rpm. Further analysis was done by using the supernatant as the crude enzyme source.

Enzyme assay: The lipase activity from the enzyme extract was measured by the olive oil hydrolysis [15]. The activity of extracellular lipase was measured in U. One unit (U) of enzyme is defined as "the amount of enzyme which releases 1 μmole of fatty acids per 1 g of substrate in 1 min under specified assay condition".

Statistical analysis: All the treatment effects were compared by the least significant difference method (spss-10-6, version-4.0, USA) after Snedecor and Cochran [16]. Significant difference among the replicates has been presented as Duncan's multiple ranges in the form of probability (p) values.

Results and Discussion

Screening of yeast cultures using various agro-industrial by-products for lipase production

Different yeast cultures such as *Candida utilis* NRRL-Y-900, *Candida lipolytica* NRRL-Y-1095, *Candida tropicalis* NRRL-Y-1552 and *Saccharomyces cerevisiae* IIB-1 were employed individually using various agro-industrial by-products such as canola meal, almond meal, coconut oil cake, soybean meal, barley bran and wheat bran for enzyme production in 250 ml Erlenmeyer flasks (Fig 1A). However, the maximum enzyme activity (1.12±0.09 U) was exhibited when soybean meal was incubated after inoculating with *C. utilis* NRRL-Y-900. Soybean meal has been considered as a best fermentable substrate for the microbial lipase production and used as a good nutrient source [17, 18]. It is thought to be a rich source of amino acids, mostly therionine, lysine and tryptophan and protein [19].

Effect of level of solid substrate

The level of solid substrate was also varied from 5-50 g (Fig 1B). Maximum enzyme activity (1.38±0.14 U) was achieved at 20 g of substrate because it is sufficient for the penetration of yeast cells and it provides an ample amount of important nutrients such as nitrogen and carbon that is essential to achieve yeast growth as well as biosynthesis of the enzyme [20, 21]. In contrast to our result, Mahadik et al [22] reported that 10 g wheat bran was optimal for enzyme synthesis.

Effect of particle size

Size of substrate particle in lipase production is a critical factor. The particle size was varied from 1.5-3.5 mm (Fig 1C). Maximum enzyme activity (1.48±0.17 U) was obtained at 3 mm of substrate particle size because smaller size of substrate particle caused poor growth of the microbes due to the substrate stickiness and reduced substrate porosity and consequently less oxygen transfer rate. On the other hand larger substrate particles retain more space between them and result in better aeration of the culture, as also reported by other workers [7]. Although larger particle size provides better aeration due to increase inter particle space, it reduces the surface area for microbial growth [23].

Selection of the appropriate diluent and substrate to diluent ratio

The moisture content is a critical factor in solid-state fermentation. Its importance for microbial growth and thereby enzyme production has been well established. Different diluents including distilled water, 0.01 N HCl, saline water, Vogel's medium (pH 5.5), phosphate buffer (pH 6) and sodium acetate buffer (pH 5), were added at a level of 20ml/20g substrate. Higher enzyme activity was observed i.e. 1.75±0.17 U with phosphate buffer (Fig 2A). It might be due to the reason that phosphate buffer caused the release of the enzyme probably by increasing the permeability of cell membrane [17]. A related work has been reported by Razak et al [24].

To check the influence of moisture on lipase production during SSF, Soybean meal was moistened with different amounts of phosphate buffer of pH 6 prior to fermentation. Higher enzyme activity of 2.16±0.13 U was attained by using 2:5 substrate to diluent ratio (Fig 2B). In contrast, Vardanega et al [25] reported 55% moisture level for the enzyme production. Enzyme synthesis declined with increase in moisture level because it reduced substrate porosity along with decrease in exchange of gas [26]. In order to improve the mass transport of nutrients in the fermentation system, it is generally beneficial to keep the level of water content just below the WHC of the solid substrate. The optimal moisture level (50%) obtained in this study was markedly lower than the WHC for soybean meal (90%).

Effect of pH

In order to determine the optimum pH for growth and lipase production by *Candida utilis* NRRL-Y-900, it was grown in the production medium at various pH in the range of 5.5-8 (Fig 2C). According to our results, the maximum enzyme activity was achieved at pH 6.5, which is optimum for *Candida* sp. to produce maximum enzyme [27]. Brozzoli et al [28] reported the same outcomes for maximum lipase production from yeast. This might be due to the reason that pH affects enzyme structure, catalytic activity of groups present in the active site of enzyme and binding of the substrate [29]. The pH of the culture changes due to the metabolic activities of microbes [30].

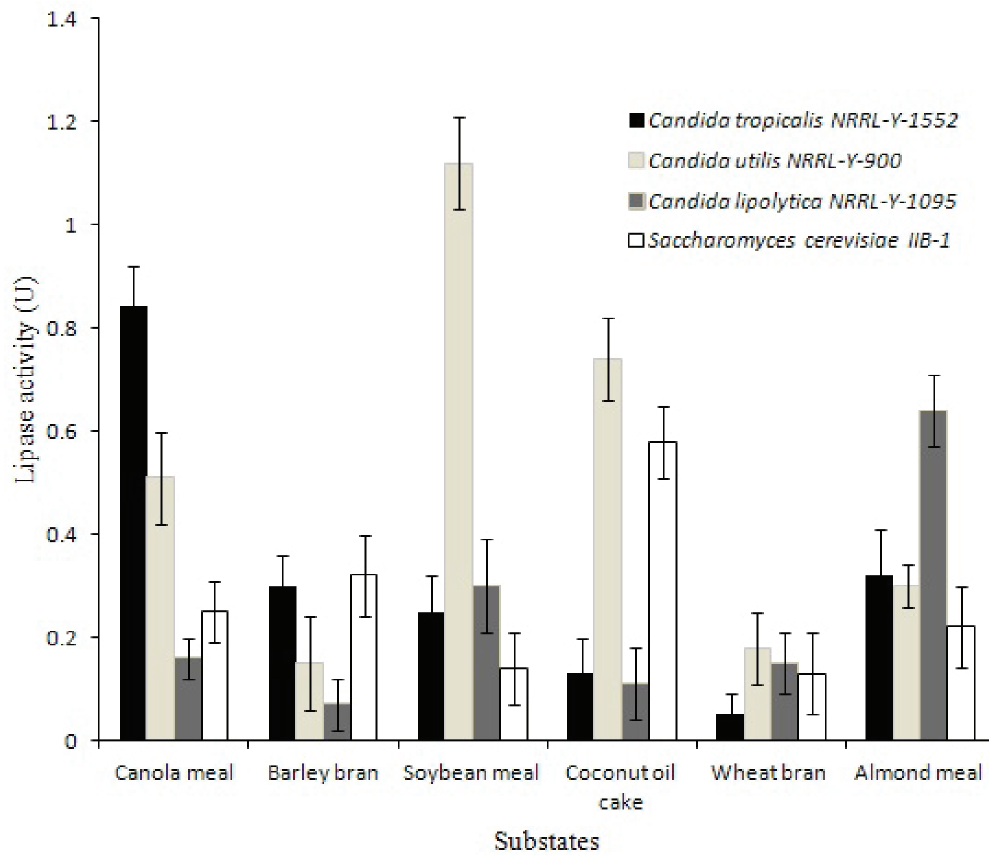

Fig 1A. Screeninng of yeast cultures by using various substrates on lipase production. The values differ significantly at a level of p≤0.05.

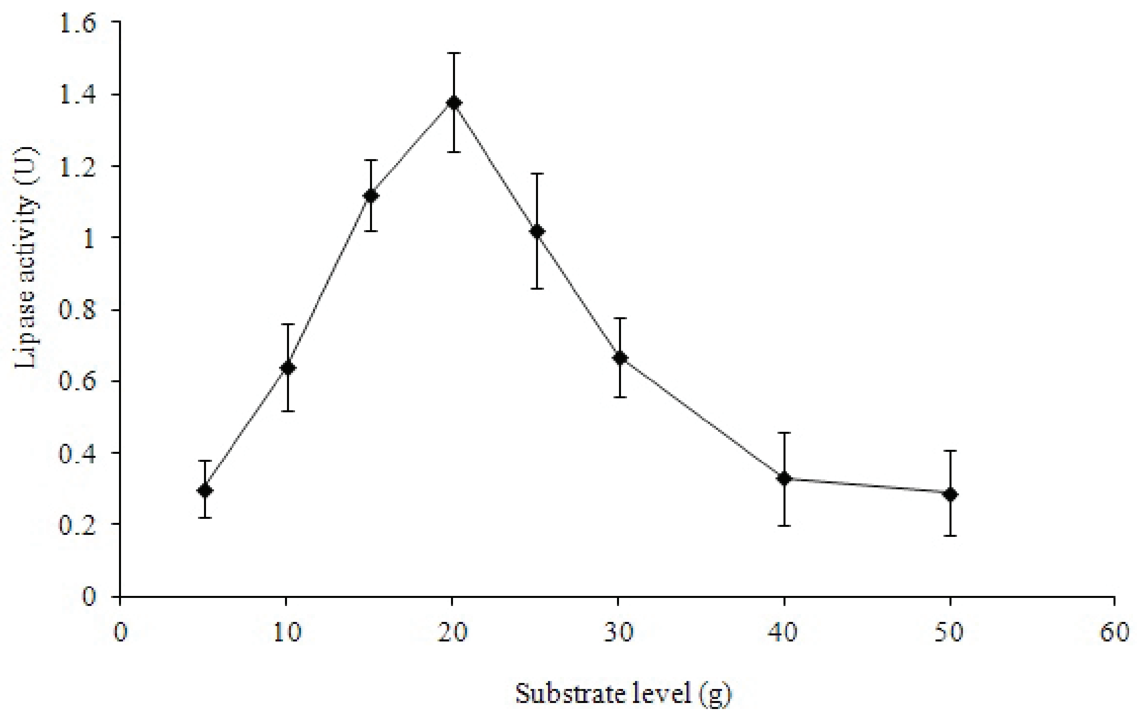

Fig 1B. Effect of different level of substrate on lipase production. The values differ significantly at a level of p≤0.05.

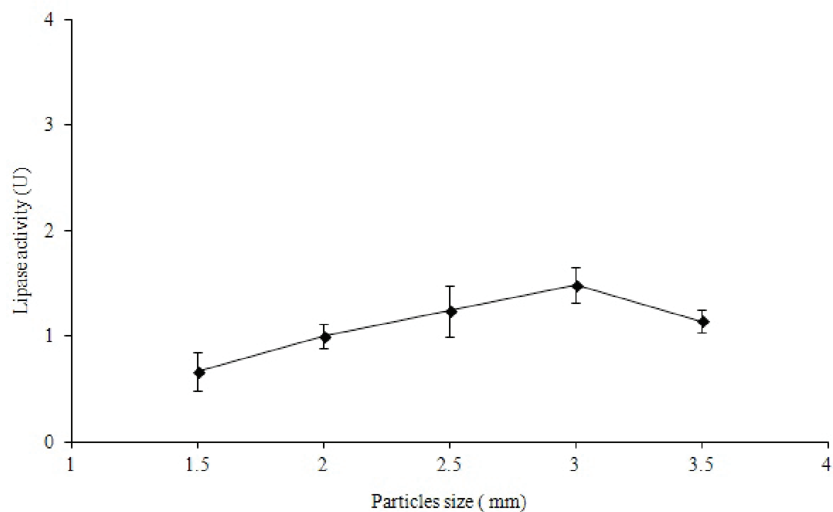

Fig 1C. Effect of different particle size on lipase production. The values differ significantly at a level of $p \leq 0.05$.

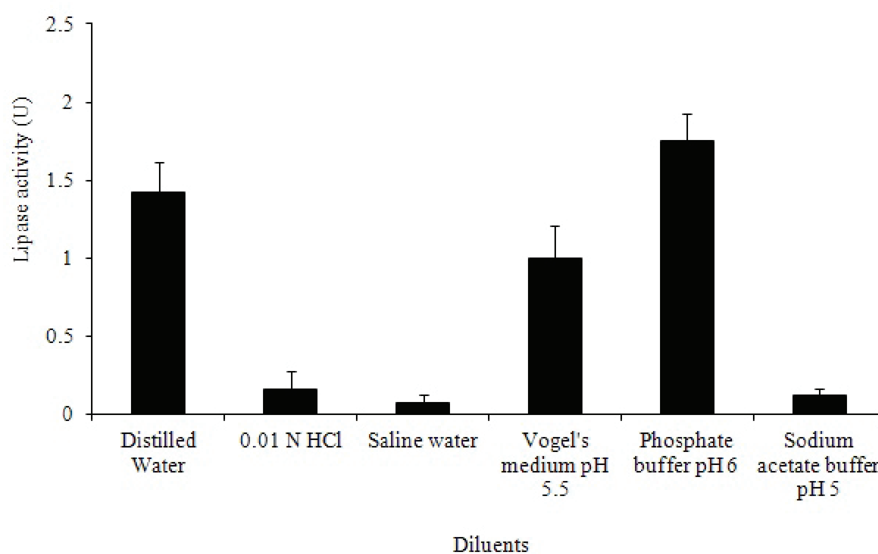

Fig 2A. Effect of various diluents on lipase production. The values differ significantly at a level of $p \leq 0.05$.

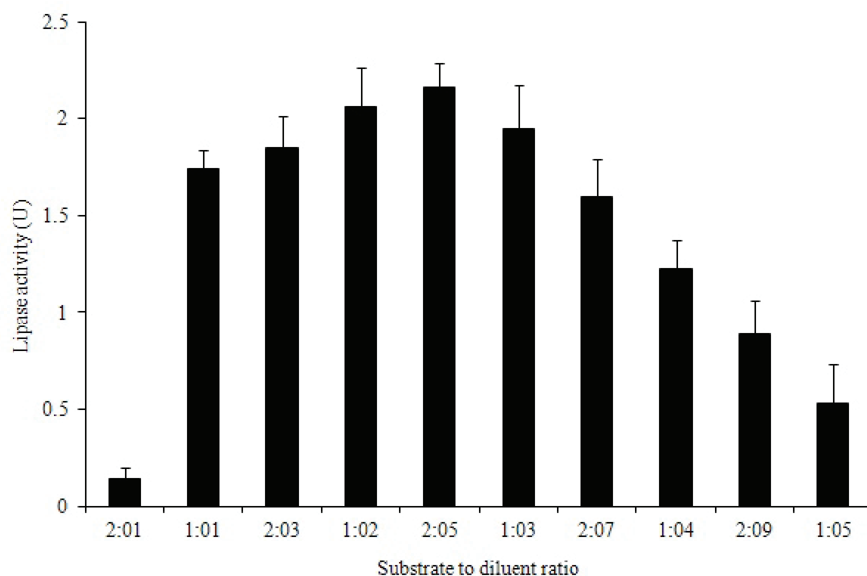

Fig 2B. Effect of substrate to diluents ratio on lipase production. The values differ significantly at a level of $p \leq 0.05$.

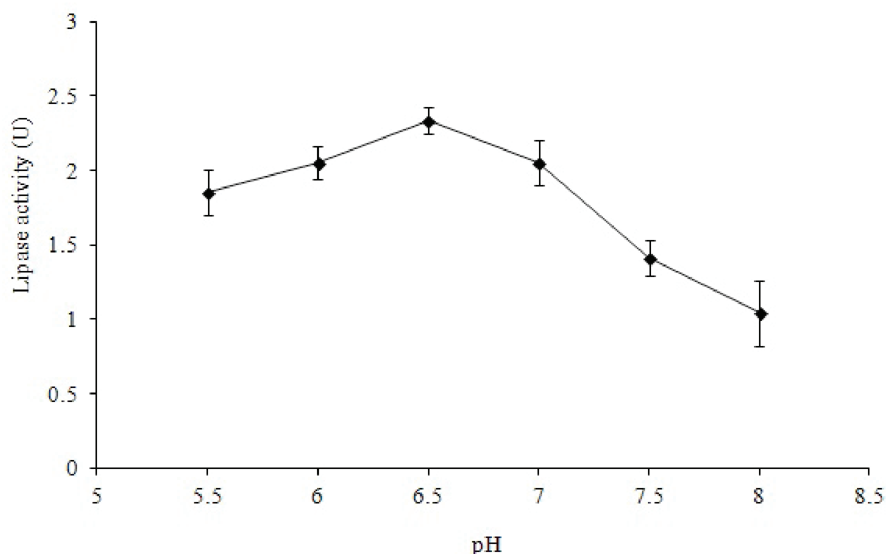

Fig 2C. Effect of pH on lipase production. The values differ significantly at a level of p≤0.05.

Effect of incubation period

The amount of lipase produced was observed after every 12 h till 144 h (Fig 3). Enzyme synthesis enhanced as the incubation period increased from 24-48 h. The maximum lipase activity i.e. 2.53±0.29 U was observed at 60 h of incubation. It could be explained by the fact that yeast was in its late exponential or early stationary phase of growth. Corzo and Revah [27] also reported maximum enzyme production at 60 h by *Yarrowia lipolytica*. It should be observed that incubation time beyond this optimum period did not lead to higher enzyme production which might be due to the accumulation of toxic compounds and lack of essential nutrients required for the growth of microorganism.

Effect of temperature

Temperature is one of the most important environmental factors affecting the enzyme production and cell growth. Incubation temperature was varied from 20-37ºC (Fig 4). At 30ºC the maximum enzyme activity (2.70±0.32 U) was observed. Enzyme activity declined, as the temperature was gradually increased from 30ºC because higher temperature affects the metabolic activity of microbial cells [23]. It was also observed that beyond this optimum temperature, the water content in the medium was reduced which badly affected the synthesis of enzyme and microbial growth. The enzyme also gets denatured as also observed by other workers [31, 32].

Effect of age of yeast culture and size of inoculum

Effect of age of yeast culture and inoculum size on lipase production was studied by *C. utilis* NRRL-Y-900. Age of yeast culture was varied from 24-72 h and level of inoculum was varied from 2.5-15% (Fig 5). The maximum activity i.e. 3.14±0.08 U was achieved when 7.5 % of 24 h old inoculum level was employed. In contrast to our work, Mladenoska and Dimitrovski [33] reported that maximum enzyme activity was obtained at 5 % (48 h old culture) inoculum of *Geotrichum candidum*. Activity of enzyme gradually declined with increase in inoculum level. It might be due to the fact that as the number of cells increased, the excess substrate was consumed for growth, hence synthesis of enzyme decreased [34].

Effect of nitrogen sources

Nitrogen sources, including organic nitrogen sources and inorganic nitrogen sources, play an important role in the synthesis of enzymes (Fig 6A). Meat extract (2%, w/v) was found to be the best nitrogen source for lipase production in this study. It might be due to the fact that the meat extract contains plenty of mineral ions, kreatin, purine bases, ammonia, phosphoric acid and potash that might have stimulated the enzyme biosynthesis. However, Corzo and Revah [28] observed that employing urea achieved maximum lipolytic activity. Among inorganic nitrogen sources used, ammonium sulphate (0.4%, w/v) was the optimal inorganic nitrogen source, which increased lipase activity (Fig 6B) because active site of enzyme might be influenced by ammonium sulphate or the ammonium ions [35]. The same result was reported by Tan et al [36].

Effect of divalent cations

Besides coenzymes, certain enzymes require a metal ion for their maximum activity (Fig 7). In the present study, it was observed that Fe+2 and Ca+2 were beneficial for biosynthesis of lipase because these divalent cations stabilize the tertiary structure of enzyme by acting

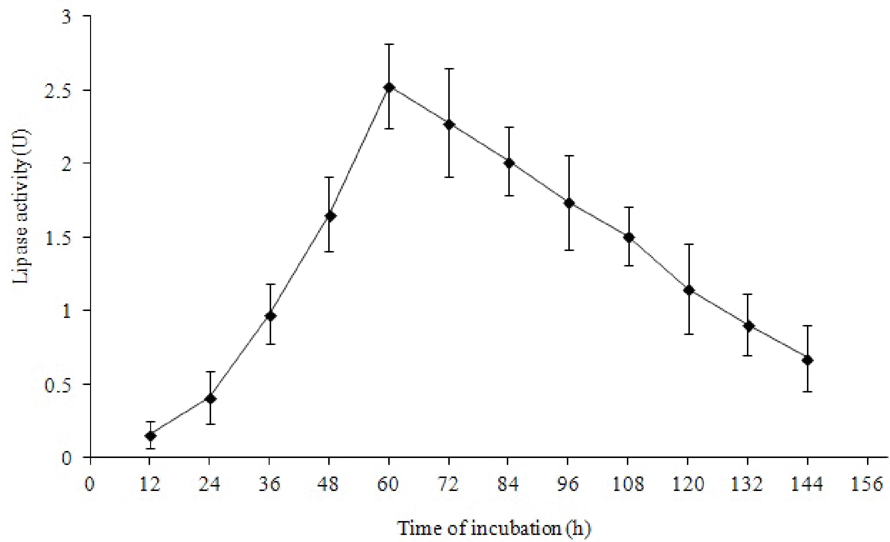

Fig 3. Effect of incubation period on lipase production. The values differ significantly at a level of p≤0.05.

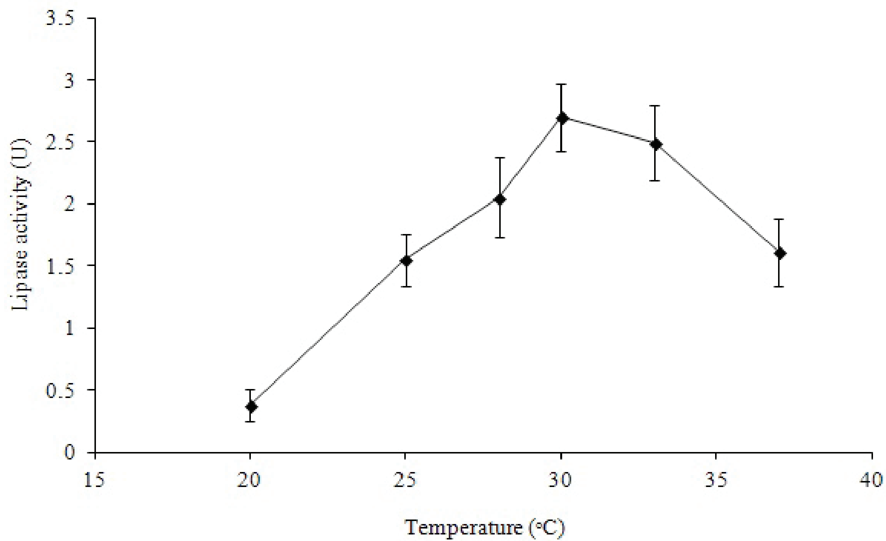

Fig 4. Effect of temperature on lipase production. The values differ significantly at a level of p≤0.05.

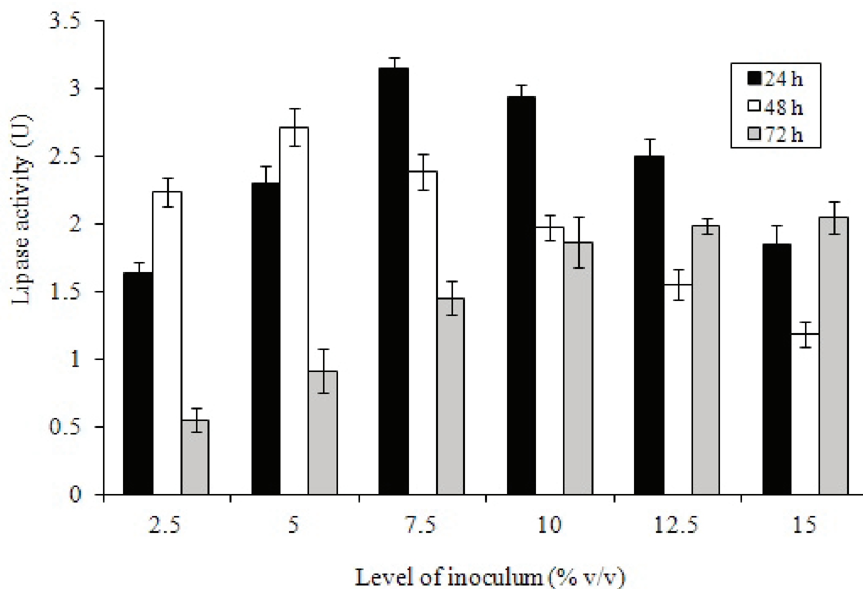

Fig 5. Effect of age and level of inoculum on lipase production. The values differ significantly at a level of p≤0.05.

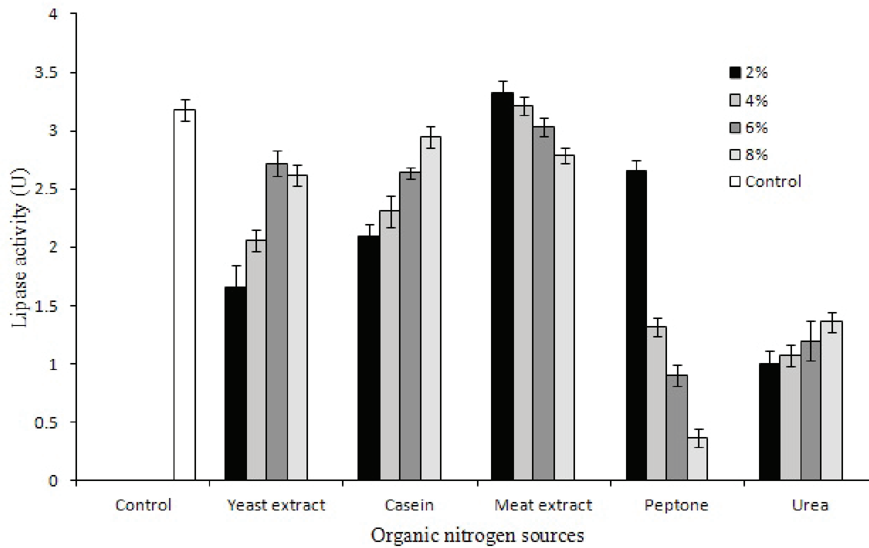

Fig 6A. Effect of different organic nitrogen sources on lipase production. The values differ significantly at a level of p≤0.05.

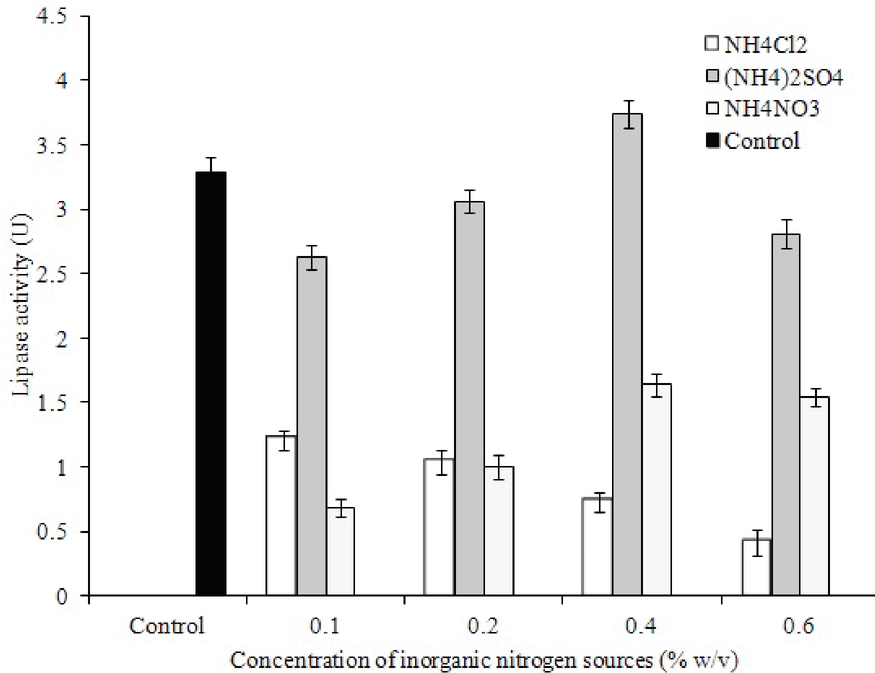

Fig 6B. Effect of different inorganic nitrogen sources on lipase production. The values differ significantly at a level of p≤0.05.

as cofactors, where their presence enhances the catalytic efficiency of the enzyme [37]. It was also observed that enzyme production was least effective when Mn+2 was added in the culture medium. The work conducted by Yamane et al. [38] is in consistent with the present report.

Conclusion

C. utilis NRRL-Y-900 was found to be better producer of extracellular lipase using soybean meal as basal substrate. Lipase production was enhanced under optimized condition. Later, it was observed that supplementation of various divalent cations and nitrogen sources (both organic and inorganic) exhibited overall variation in the level of enzyme accumulation. In summary, *C. utilis* NRRL-Y-900 has been found to be a potential candidate for lipase production at industrial scale because of its ability to utilize agro-industrial by-products in solid state fermentation.

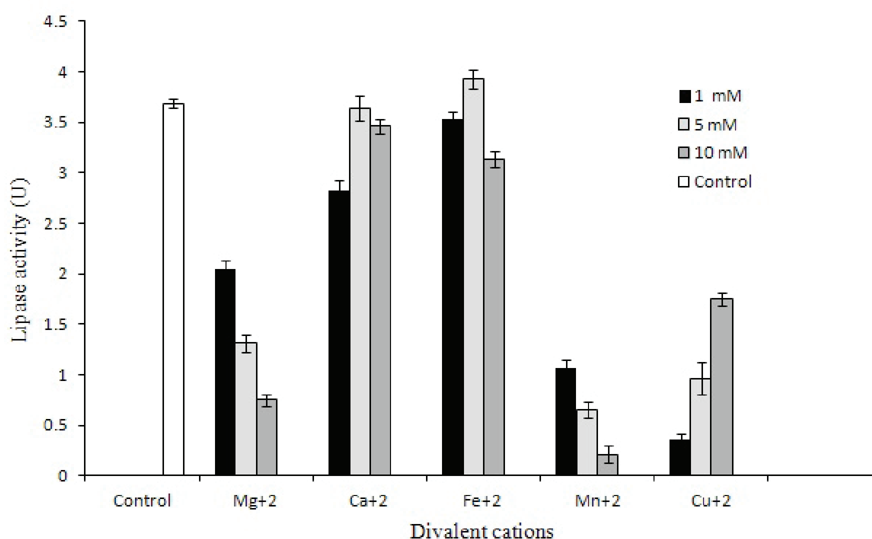

Fig 7. Effect of divalent cations on lipase production. The values differ significantly at a level of $p \leq 0.05$

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